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**Citation for published version:**

Pavitt, AT, Walling, CA, Möstl, E, Pemberton, JM & Kruuk, LEB 2015, 'Cortisol but not testosterone is repeatable and varies with reproductive effort in wild red deer stags', *General And Comparative Endocrinology*. <https://doi.org/10.1016/j.ygcen.2015.07.009>

**Digital Object Identifier (DOI):**

[10.1016/j.ygcen.2015.07.009](https://doi.org/10.1016/j.ygcen.2015.07.009)

**Link:**

[Link to publication record in Edinburgh Research Explorer](#)

**Document Version:**

Publisher's PDF, also known as Version of record

**Published In:**

General And Comparative Endocrinology

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Contents lists available at ScienceDirect

## General and Comparative Endocrinology

journal homepage: [www.elsevier.com/locate/ygcen](http://www.elsevier.com/locate/ygcen)

## Cortisol but not testosterone is repeatable and varies with reproductive effort in wild red deer stags

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## ARTICLE INFO

## Article history:

Received 16 November 2014

Revised 9 July 2015

Accepted 21 July 2015

Available online xxxx

## Keywords:

Androgens

Biological assay validation

Dominance

Faecal hormone metabolites

Glucocorticoids

Repeatability

Rut

Seasonal cycles

## ABSTRACT

Although it is known that hormone concentrations vary considerably between individuals within a population, how they change across time and how they relate to an individual's reproductive effort remains poorly quantified in wild animals. Using faecal samples collected from wild red deer stags, we examined sources of variation in faecal cortisol and androgen metabolites, and the potential relationship that these might have with an index of reproductive effort. We also biologically validated an assay for measuring androgen metabolites in red deer faeces.

We show that variation in hormone concentrations between samples can be accounted for by the age of the individual and the season when the sample was collected. Faecal cortisol (but not androgen) metabolites also showed significant among-individual variation across the 10-year sampling time period, which accounted for 20% of the trait's phenotypic variance after correcting for the age and season effects. Finally, we show that an index of male reproductive effort (cumulative harem size) during the mating season (rut) was positively correlated with male cortisol concentrations, both among and within individuals. We suggest that the highest ranking males have the largest cumulative harem sizes (i.e. invest the greatest reproductive effort), and that this social dominance may have associated behaviours such as increased frequency of agonistic interactions which are associated with corresponding high levels of faecal cortisol metabolites (FCM).

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## 1. Introduction

Although hormone concentrations vary between individuals within a population (Williams, 2008), how this relates to individual-level variation in fitness-related behaviour remains poorly quantified in the wild. To date, work in this area has been dominated by laboratory and captive populations (e.g. Bartos et al., 2010; Ketterson and Nolan, 1992), where variation in hormone levels and/or behaviours may not be representative of that seen in wild systems (Bartos et al., 2010). In this study we focused on variation in male behaviour during the mating season (rut) in a wild population of red deer (*Cervus elaphus*), and tested for associations with faecal concentrations of androgen and glucocorticoid metabolite. Red deer stags exhibit dominance hierarchies

throughout the year (Bartos et al., 2010; Lincoln et al., 1972), culminating in peak male–male agonism during the rut (Lincoln et al., 1972) when dominance status determines access to harems of females (Clutton-Brock et al., 1982). In this paper, we use data from a long-term study of a wild red deer population to test for associations between cumulative harem size (an index of reproductive effort which indicates access to females during the rut) and androgen and glucocorticoid levels respectively.

Androgen concentrations do not remain consistent across individual males' lifetimes, but vary within and between years in association with behavioural changes (Book et al., 2001 and references therein, Lynch et al., 2002; Wingfield et al., 1990). Within a year, seasonal variation in testosterone concentrations often correlates with reproductive cycles and associated changes in male–male conflict (Lynch et al., 2002; Wingfield et al., 1990), peaking during the breeding season (September–November in our study population) when male aggression is at its height (e.g. Lincoln et al., 1972; Pereira et al., 2005). Where there is substantial age-related variation in reproductive effort, androgen concentrations might

Abbreviations: FAM, faecal androgen metabolites; FCM, faecal cortisol metabolites.

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<http://dx.doi.org/10.1016/j.ygcen.2015.07.009>

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also be expected to vary with age (Book et al., 2001 and references therein). Red deer stags show considerable variation in reproductive effort and output across their lifetime (Nussey et al., 2009). Stags in their reproductive prime tend to engage in more aggressive encounters than younger and older individuals (Clutton-Brock et al., 1979), and therefore might also be expected to exhibit higher testosterone concentrations overall (as has been shown in other deer species: Bubenik and Schamsa, 1986). Links between testosterone concentrations and male fitness-related traits are well established in several taxa (see reviews by Hau, 2007 and Wingfield et al., 2001) including red deer (Lincoln et al., 1972; Malo et al., 2009). Less is known, however, about the potential relationship between testosterone and behavioural investment in reproduction. Red deer stags exhibit dominance hierarchies throughout the year (Bartos et al., 2010; Lincoln et al., 1972), which determines their access to females during the rut (Clutton-Brock et al., 1982) and thus their chances of siring offspring conceived in that year. Given positive relationship between testosterone and social rank in this species (Bartos et al., 2010), testosterone levels might be expected to show a positive relationship with the size or length of time harems are held for (i.e. measures of reproductive effort), and through that, with a stag's annual reproductive success (Appleby, 1982; Gibson and Guinness, 1980).

Expectations for cortisol are somewhat more complex. Cortisol is the dominant circulating glucocorticoid in red deer (Ingram et al., 1999), and is generally (across taxa) highest when animals are exposed to unpredictable or uncontrollable stressors (Greenberg et al., 2002), although there is considerable individual variation in baseline levels. Where observed, circannual cycles in cortisol concentration are likely to reflect seasonal variation in stressors, such as challenging climatic conditions (e.g. low temperature: Huber et al., 2003a) or social instability (e.g. male conflict during the breeding season: Strier et al., 1999). Males investing greater effort in reproduction might also have higher levels of cortisol if that effort is associated with energetic or physiological costs (e.g. the Cort-Adaptation Hypothesis: Bonier et al., 2009), or if this is an adaptive response which enables them to maximise their fitness in unpredictable environments (Boonstra, 2013). Given that energetic investment in reproduction peaks during middle-age in red deer stags (Nussey et al., 2009), individuals might also be expected to have higher levels of cortisol during their reproductive prime. Evidence, however, suggests that this might be confounded by the physiological effects of ageing, which can see circulating cortisol levels increasing with age due to desensitisation of the cortisol feedback loop (Sapolsky, 1991; van Cauter et al., 1996).

Similarly, it is also difficult to predict the association between cortisol levels and behavioural investment in reproduction. If the maintenance of social dominance (a trait closely associated with reproductive opportunity Clutton-Brock et al., 1982) involves greater aggression and energetic investment (Clutton-Brock et al., 1979; Lincoln et al., 1972), then males investing the most in reproduction might be expected to have higher cortisol levels as a result (e.g. Muller and Wrangham, 2004). This scenario would also predict positive associations between androgens and cortisol. The alternative hypothesis is that baseline glucocorticoid levels would be highest in animals with lower relative fitness (e.g. the Cort-Fitness Hypothesis: Bonier et al., 2009), due, for example, to poorer quality or suppressed reproductive systems (Liptrap, 1993). If high cortisol concentrations were linked to reduced quality and fitness, then individuals with high levels might also be expected to die at a younger age, leading to a population-level decline in cortisol concentrations amongst older age classes (van de Pol and Verhulst, 2006).

In this study, we quantify (a) the effects of season and age on variation in faecal androgen and cortisol metabolite concentration; and (b) the relationships between concentrations of these

hormones and cumulative harem size (an index of male reproductive effort) during the breeding season. For this, a large dataset at the individual-level is required, making the wild red deer on the Isle of Rum National Nature Reserve (NNR) in Scotland an ideal study population as life history, behaviour and reproductive data have been collected from individually identifiable deer since 1972 (Clutton-Brock et al., 1982).

## 2. Methods

### 2.1. Faecal sample collection

Faecal samples were collected from individually identifiable wild red deer stags in the North Block study area of the Isle of Rum NNR, Scotland (see Clutton-Brock et al., 1982 for full description of the study population and site) between 2004 and 2013 (see Fig. S1 for the distribution of repeat sampling between individuals). Fresh faecal samples were collected both opportunistically and from targeted collection sessions within 5 min of witnessing defecation, and only from positively identified individuals. They were stored at  $-20^{\circ}\text{C}$  in a field freezer (mean time from collection to freezing:  $101 \text{ min} \pm 10 \text{ SE}$ ), before being packed in ice and returned to laboratory freezers where they were kept at  $-20^{\circ}\text{C}$  until extraction.

### 2.2. Faecal steroid extraction

Individual faecal samples were fully defrosted and homogenised to evenly distribute hormones throughout faeces. Once homogenised, 0.5 g of wet sample was extracted with 5 ml of methanol (90%), gently shaken (overnight at  $20^{\circ}\text{C}$ ) and centrifuged (20 min at 652 g), after which 1 ml of the resulting supernatant was transferred to a clean tube and stored at  $-20^{\circ}\text{C}$  until assay. Faecal samples ( $n = 194$ ) were collected from 73 individuals who were either born in the study area ( $n = 53$  males) or were visiting males born in other parts of the island ( $n = 20$  males).

### 2.3. Faecal hormone immunoassays

Concentrations of faecal androgen and cortisol metabolite (FAM and FCM respectively) were measured using group-specific enzyme immunoassays (EIAs). Both assays were carried out following the same established methods (Huber et al., 2003b; Palme and Mostl, 1994) with group-specific antibodies.

Androgens are extensively metabolised before excretion, mainly in the liver. As the main testosterone metabolites are unknown in red deer, no immunoassay has previously been validated for FAM in this species. We therefore first biologically validated a suitable assay by testing the ability of three androgen assays (which measured androgen metabolites with a  $17\beta$  hydroxy-group, a  $17\alpha$ -hydroxy-group, or a  $17$ -oxo-group), to detect biologically meaningful differences (see Supplementary Information 2). This was biologically validated because, being a wild population, invasive procedures (e.g. chemical manipulations) were not possible (an approach outlined in Palme, 2005). Of the assays tested, the  $17$ -oxo-androgen EIA both had the greatest reactivity, showing that most of the immune-reactive FAMs were excreted in this form, and best discriminated between sexes and male reproductive status in our study population (see Supplementary Information 2 for comparison of the assays tested). This test has previously been used successfully to measure FAM concentrations in other mammal species, including ungulates (Ganswindt et al., 2002; Hoby et al., 2006). Faecal cortisol metabolite (FCM) levels were measured using a group-specific  $11$ -oxoetiocholanolone EIA which has previously been validated

in red deer using both ACTH (adrenocorticotrophic hormone) challenge and natural disturbance tests (Huber et al., 2003b). These immunoassays followed previously published methodology (described in Huber et al., 2003b; Palme and Mostl, 1994), but with Protein A used for the first coating of the microtiter plates instead of affinity purified anti-rabbit IgG.

Serial dilutions of 24 pooled samples showed high parallelism with the standard curve in both hormone groups ( $p < 0.001$ ), which had limits of detection (LOD) of 0.89 ng/g faeces for the FAM assay and 3.51 ng/g faeces for the FCM assay. The intra- and inter-assay coefficients of variance (CV) were calculated at 4.85% and 20.64% for FAM and 4.01% and 22.65% for the FCM assay. Several assay plates were run per day, and given that previous studies have found assay date to account for significant variation between samples (Pavitt et al., 2014), the mean within-day inter-assay CV was also calculated. This gave a mean within-day inter-assay CV of 12.46% ( $\pm 1.80$  SE) for FAM and 15.02% ( $\pm 3.72$  SE) for FCM. From the original 194 samples, 19 FAM and 16 FCM measures were removed due to low repeatability of concentrations measured between duplicates (CV > 10%). A further 34 FAM measures were removed because they fell below the LOD (removal of these 34 low FAM measures did not affect the results of the model, see [Supplementary Information 3](#) for details).

#### 2.4. Index of reproductive effort: cumulative harem size

Red deer are a polygynous species in which males compete for harems of females during the breeding season (Clutton-Brock et al., 1982). In this study, cumulative harem size was used as a proxy for annual male reproductive effort, and measured as a stag's total number of "hind-days held". Thus cumulative harem size was defined as the sum of a male's daily harem size across the rutting period (15th September–15th November) for a given year, based on daily censuses taken during this period. Censuses recorded male–female associations, and used proximity and behaviour to assign females to a stag's harem. Stags holding harems outside of the North Block study area of the Isle of Rum NNR were not recorded. By combining a male's harem size on a particular day and the number of days on which he held a harem, cumulative harem size is a good measure of total investment in reproduction in a given year. Previous analyses have shown this measure to be closely linked to both social rank and reproductive success in males (Appleby, 1982; Pemberton et al., 1992). These analyses used records of harem holding collected between 1971 and 2013, comprising of 2833 measures of cumulative harem size from 815 males (mean: 3.48 measures/stag  $\pm 0.01$  SE).

### 3. Statistical analysis

A multivariate ("multi-response") mixed model was fitted to the data in ASReml-R ver.3.0.3 (package: asreml, Butler, 2009) to explore potential causes of variation in, and covariance between, FAM, FCM and cumulative harem size (CHS). All three measures were log-transformed to normalise residuals.

This multivariate model therefore had three response variables, and the structure:

FAM, FCM, CHS  $\sim$  trait-specific\_fixed\_effects + (individual ID) + (year) + (residuals).

The trait-specific fixed effects are discussed in Section 3.1 below, and the random effects (in parentheses) in Section 3.2.

Although FAM and FCM concentrations were only available for a subset (2004–2013) of the individuals for whom we had measures of cumulative harem size, all individuals with observations of cumulative harem sizes (1971–2013) were included in the multivariate models, with missing values for FAM and FCM where

necessary. Inclusion of these individuals improves the accuracy of estimation of the variance components associated with cumulative harem size. Further, improved information on the distribution of cumulative harem size will both improve the accuracy and reduce the uncertainty (SE) of estimation of any covariance between cumulative harem size and FAM or FCM. As outlined below, we estimated these covariances at both among-individual and within-individual (i.e. residual) individual levels.

Our analyses, therefore, used a total of 141 FAM measures (from 66 stags), 178 FCM measures (from 67 stags) and 2833 measures of cumulative harem size (from 815 stags). Of these, 105 measures of cumulative harem size had corresponding FAM concentrations for a given stag in a given year, and 138 had corresponding FCM concentrations. A further 33 FAM and 43 FCM concentrations were also included for males which were either below rutting age (<4 years old; FAM:  $n = 23$  from 13 deer; FCM:  $n = 32$ , from 12 deer), or did not hold a harem within the study area in the year of sample (FAM:  $n = 10$  from 5 deer; FCM:  $n = 11$  from 5 deer). Where males had repeat measures of hormone concentration in a given year, the sample collected closest to the start of their harem-holding period was associated with their cumulative harem size for that year. This allowed estimation of the residual covariance between cumulative harem size and hormone concentration. These models therefore included 71 FAM concentrations and 82 FCM concentrations that had a corresponding cumulative harem size, although all measures of hormone concentrations were included in the analyses (FAM:  $n = 141$ , FCM:  $n = 178$ ).

#### 3.1. Fixed effects

Age at the time of sampling (in years) was fitted as a fixed effect for all three response variables. A quadratic term for age was also tested because a number of male reproductive traits are known to have a quadratic relationship with age in this population (Nussey et al., 2009). This was retained in the model for FAM and cumulative harem size, but not for FCM, for which it was not significant ( $p = 0.541$ ). Sample month (11-level factor for January–November) and the age at final sampling were also included for both hormone concentrations. Age at final sampling was fitted to test for the 'selective disappearance' of particular hormone phenotypes with age, allowing us to distinguish between within-individual and population-level changes (van de Pol and Verhulst, 2006). The date of assay (7-level factor) was also included for both hormone concentrations as previous studies have found assay date to account for significant variation amongst samples, possibly due to fluctuations in laboratory temperature (Pavitt et al., 2014). Time of sample collection (all samples were collected between 09:15 and 21:10), and time (in minutes) from sample collection to freezing (mean time: 96 min  $\pm 9$  SE, range: 2–391 min) were also tested for effects on FAM and FCM concentrations, as both have been shown to affect hormone concentrations (Ingram et al., 1999; Suttie et al., 1992). There was not, however, a significant effect of either collection time (FAM: Est. =  $0.002 \pm 0.022$  SE,  $p = 0.836$ ; FCM: Est. =  $0.008 \pm 0.010$  SE,  $p = 0.801$ ) or time to freezing (FAM: Est. <  $0.001 \pm 0.002$  SE,  $p = 0.780$ ; FCM: Est. <  $0.001 \pm 0.001$  SE,  $p = 0.891$ ), and so both were excluded from the final model. Fixed effects were tested for significance using incremental Wald tests, and the optimal model was accepted when all remaining fixed effects were significant at  $p < 0.05$ .

#### 3.2. Components of variance and covariance between hormone production and cumulative harem size

Individual identity ( $n = 815$ ), year of sampling ( $n = 42$ ), and unexplained residual effects were fitted as random effects for all three traits in the model. After comparing nested models fitted



with and without year of sampling, this random effect was excluded from the final model because it did not significantly affect any of the three traits (FAM:  $p = 0.945$ , FCM:  $p = 0.720$ , cumulative harem size,  $p = 0.492$ ; Table S5(b)). The repeatability of all three traits was estimated as the proportion of that trait's overall phenotypic variance that was accounted for by individual identity (i.e. among-individual differences).

After testing the variances associated with individual identity and residual effects, covariances between the respective random effects were also fitted to explore relationships between the three traits at both individual and residual levels. In order to test the significance of covariances, we used likelihood ratio tests (LRT) to compare the full model with models where each particular covariance was constrained to 0 in turn. The LRT assumed the difference in the likelihood of the two models was a chi-squared distribution with 1 degree of freedom. Because no individual-level variation was found in FAM concentrations when fitting a multivariate model with just variance components ( $p = 0.664$ , Table S5(a)), we did not attempt to estimate individual-level covariances between FAM and both FCM and cumulative harem size; these parameters were therefore fixed at 0 in the final model. This model was not a significantly worse fit to the data than a model in which these covariances were estimated (LRT:  $X^2_{(2)} = 0.451$ ;  $p = 0.637$ ), but is more statistically justified than estimating the covariance between two parameters when there is no robust statistical evidence of any significant variance in one of them. In the final model, therefore, the only testable (i.e. non-zero) among-individual covariance was between FCM and cumulative harem size.

#### 4. Results

Both faecal androgen and cortisol metabolite concentrations varied substantially between samples. Concentrations of FAM ranged from 2.7–17216.3 ng/g faeces (mean concentration: 447.0 ng/g faeces  $\pm$  154.9 SE), and FCM from 5.3–680.9 ng/g faeces (mean concentration: 61.5 ng/g faeces  $\pm$  6.3 SE). Measures of cumulative harem size also varied considerably, ranging from 1–646 hind-days held (mean: 56.2 hind-days held  $\pm$  1.5 SE).

##### 4.1. Seasonal and age effects

Concentrations of both hormones showed significant variation with month ( $p < 0.001$ ; Table 1; Fig. 1). FAM levels peaked in September, decreased through October and overall remained low for the rest of the year (Fig. 1). FCM concentrations also increased during the autumn period (with peak concentrations September–October), but showed an additional peak in February–March (Fig. 1). FAM, FCM and cumulative harem size also varied significantly with a stag's age (FAM:  $p < 0.001$ ; FCM:  $p = 0.012$ ; cumulative harem size:  $p < 0.001$ ; Table 1; Fig. 2 and Fig. s5), however age at final sampling did not significantly improve the model when considered for either hormone (FAM:  $p = 0.777$ ; FCM:  $p = 0.864$ ; Table 1). FAM concentrations increased with age until around 8–9 years old, after which they began to decline (Fig. 2(a)). In accordance with previous studies of this population (Nussey et al., 2009), cumulative harem size also peaked around 8–11 years old (Fig. s5). By contrast the relationship between FCM and age was linear, with older individuals having higher concentrations (Fig. 2(b)). In agreement with previous studies (Pavitt et al., 2014), both FAM and FCM varied with assay date.

##### 4.2. Variance components

FAM levels were not repeatable among individuals ( $p = 0.719$ ; Table 2(a)), with differences between individuals only accounting

for around 3% ( $0.03 \pm 0.08$  SE) of the variance observed in this trait. In contrast, both FCM and cumulative harem size varied significantly both at the among- and within-individual levels ( $p < 0.005$ , Table 2). FCM had a repeatability of  $0.20 \pm 0.06$  SE (i.e. individual identity accounted for 20% of the variance seen in this trait after correcting for the fixed effects), and cumulative harem size had a repeatability estimate of  $0.26 \pm 0.03$  SE.

##### 4.3. Covariance between hormone levels and cumulative harem size

Stags with greater cumulative harem sizes were also likely to have higher FCM concentrations (see Fig. 3 for overall phenotypic relationship between these two variables). This positive covariance between cumulative harem size and FCM was found both at the among-individual (LRT:  $X^2_{(1)} = 3.067$ ,  $p = 0.013$ ; Table 2(a)), and at within-individual or residual (LRT:  $X^2_{(1)} = 1.876$ ,  $p = 0.049$ ; Table 2(b)) levels. Given that FAM concentrations were not repeatable amongst individuals (Table 2(a); Table S5(a)), we did not attempt to estimate any among-individual covariance between FAM and either FCM or cumulative harem size (Table 2). There were non-significant negative covariances within individuals (i.e. residual covariance) between FAM, and both FCM (LRT:  $X^2_{(1)} = 0.006$ ,  $p = 0.910$ ; Table 2(b)) and cumulative harem size (LRT:  $X^2_{(1)} = 1.139$ ,  $p = 0.131$ ; Table 2(b)).

#### 5. Discussion

This study utilised non-invasive sampling techniques to explore the factors associated with among- and within-individual variation in faecal concentrations of both androgen and cortisol metabolites in a wild population of male red deer. We found clear seasonal and age-related variation in faecal concentrations of both hormones, as well as a significant positive relationship between a stag's FCM levels and their cumulative harem size at both the among- and within-individual level. The analysis is amongst the first to test assumptions about the relationships between FAM and FCM concentrations and an index of reproductive effort in the wild.

In accordance with expectations, FAM levels were highest in the build-up to and during the reproductive season (August–October), and in prime-aged stags (aged 8–9 years old). Testosterone is known to regulate the expression of both reproductive and aggressive behaviours in red deer (Fletcher, 1978; Lincoln et al., 1972). Rutting behaviour, for example, can be eliminated by castrating a red deer stag, and restored through testosterone implants (Lincoln et al., 1972). It was therefore not surprising to observe maximum FAM levels during the rut when inter-male aggression is greatest, and at the age when male annual reproductive performance, and thus presumably agonistic interactions between competing males, peaks (Nussey et al., 2009).

We found no evidence of among-individual variance in FAM concentrations in this study. This contrasts with the limited results published for other taxonomic groups, which show significant repeatability of both plasma testosterone (lizards: While et al., 2010) and faecal androgen metabolites (Kralj-Fisher et al., 2007; Pelletier et al., 2003) in wild systems. It is worth noting, however, that these studies were either considered repeatability within the shorter time-periods of days (Pelletier et al., 2003) or months (Kralj-Fisher et al., 2007; While et al., 2010) or were based on much smaller sample sizes (Kralj-Fisher et al., 2007; While et al., 2010), than our study which collected samples over several years. The lack of any among-individual variance in FAM concentrations meant we did not examine covariances with cumulative harem size at the level of the individual. Given that sample year also explained no variance (see Methods), this lack of among-individual FAM variance could not be attributed to annual

**Table 1**

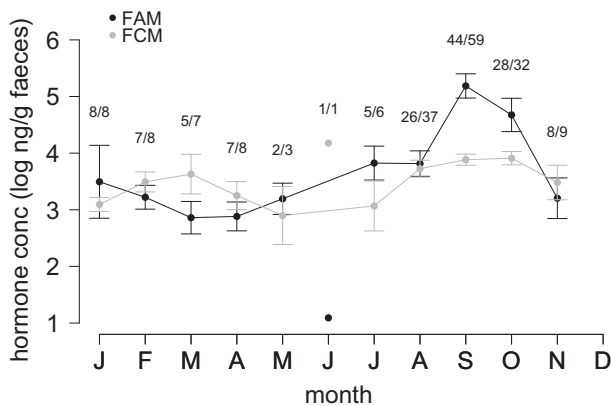
Correlates of FAM, FCM and cumulative harem size. Multivariate mixed effects model estimating the main effects of extrinsic factors on individual-level variation in (a) faecal androgen metabolite (FAM) concentrations, (b) faecal cortisol metabolite (FCM) concentrations, and (c) cumulative harem size. See Table S6 for breakdown of assay date estimates.

Fixed effects	FAM (n = 141)			FCM (n = 178)			Cumulative harem size (n = 2833)		
	Est.	SE	p	Est.	SE	p	Est.	SE	p
(Intercept)	4.888	0.923	<0.001***	3.623	0.522	<0.001***	−33.73	6.691	<0.001***
Age	0.117	0.061	0.032*	0.058	0.031	0.012*	0.174	0.009	<0.001***
Age <sup>2</sup>	−0.047	0.01	<0.001***	–	–	–	−0.038	0.002	<0.001***
February <sup>a</sup>	−0.251	0.656		0.371	0.311		–	–	
March <sup>a</sup>	−0.925	0.715		0.393	0.32		–	–	
April <sup>a</sup>	−0.386	0.653		−0.051	0.31		–	–	
May <sup>a</sup>	−0.191	1.045		0.224	0.41		–	–	
June <sup>a</sup>	−1.121	1.366		−0.179	0.664		–	–	
July <sup>a</sup>	−0.342	0.735	<0.001***	−0.088	0.339	<0.001***	–	–	
August <sup>a</sup>	0.312	0.532		0.787	0.25		–	–	
September <sup>a</sup>	1.745	0.504		0.803	0.245		–	–	
October <sup>a</sup>	1.054	0.538		0.801	0.252		–	–	
November <sup>a</sup>	−0.157	0.648		0.458	0.308		–	–	
Age at final sample	−0.019	0.06	0.777	−0.005	0.032	0.864	–	–	
Assay date <sup>b</sup>	7 estimates		<0.001***	7 estimates		<0.001***	–	–	

\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

<sup>a</sup> Estimates for month are relative to estimates of January.

<sup>b</sup> See Table S6 for complete breakdown of assay date estimates.

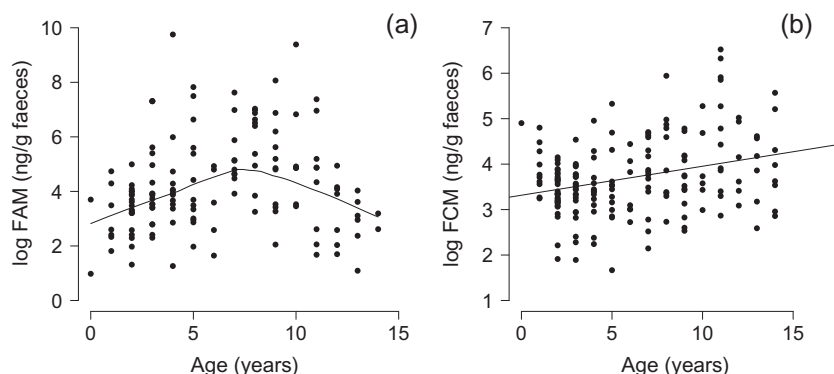


**Fig. 1.** Seasonal cycles in FAM & FCM. Variation in log transformed faecal androgen metabolite (FAM; black) and faecal cortisol metabolite (FCM; grey) concentrations with month. Points represent monthly means  $\pm$  standard errors (see Fig. S4 for seasonal variation in the fitted values after correcting for age, assay date and individual identity in univariate hormone models). Numbers represent monthly sample sizes for FAM and FCM respectively. Only one sample was collected in June and so no estimate of error is possible.

variation above the effects of age. Furthermore, whilst a positive relationship between FAM and cumulative harem size was expected (see reviews by Hau, 2007; Wingfield et al., 2001), we

did not find this to be the case. Instead there was a non-significant negative trend at the within-individual level ( $p = 0.131$ ; Table 2(b)), which is possibly more in concurrence with negative relationships between testosterone levels and dominance seen in populations during periods of social hierarchical instability (Bartos et al., 2010).

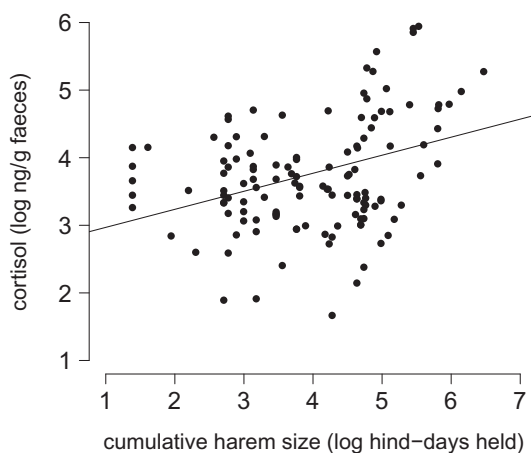
This study identified two peaks in FCM concentration across the year, coinciding with periods of high environmental or physiological stress: one during the late winter (peaking in March), and a second one during the early autumn. The first peak is similar to previous findings in captive red deer (Huber et al., 2003a): winter is known to be energetically challenging for the deer on Rum, with limited food availability and high mortality rates (Clutton-Brock et al., 1982). The second peak in FCM coincides with the rutting season, and could be the result of increased agonistic interactions between stags competing for females (see Romero and Butler, 2007 for discussion of the stress-response). Elevated cortisol levels during the reproductive season have been reported in males of other polygynous species (Lynch et al., 2002; Strier et al., 1999), although this has not previously been found when analysing seasonal variation in red deer (Huber et al., 2003a; Ingram et al., 1999). We have no explanation for this lack of consensus with other deer studies, except possibly that the previous work has focussed on captive deer which may not have been exposed to the same conditions, behaviours or social interactions as those in the wild.



**Fig. 2.** Age-related variation in FAM & FCM. Variation in log transformed (a) faecal androgen metabolite (FAM) and (b) faecal cortisol metabolite (FCM) with age. The figures show the raw data, and the smooth lines were fitted from regressions of log-transformed hormone concentrations against age.

**Table 2**  
Relationships between FAM, FCM and cumulative harem size. Multivariate mixed effects model estimating variances (diagonal), correlations (above diagonal) and covariances (below diagonal) for faecal androgen metabolites (FAM), faecal cortisol metabolites (FCM) and cumulative harem size (CHS) at (a) among-individual and (b) residual within-individual levels (SE in brackets). Shaded cells indicate values that were fixed and not allowed to vary. Statistically significant variances and covariances are in bold.

(a) Among-individual				(b) Within-individual			
	FAM	FCM	CHS		FAM	FCM	CHS
FAM	0.049 (0.154) $\chi^2 = 0.065$ $p = 0.719$	0	0	FAM	<b>1.481 (0.230)</b> $\chi^2 = 256.044$ $p < 0.001$	−0.011 (0.108)	−0.215 (0.135)
FCM	0	<b>0.184 (0.068)</b> $\chi^2 = 4.245$ $p = 0.004$	0.577 (0.182)	FCM	−0.008 (0.078) $\chi^2 = 0.006$ $p = 0.910$	<b>0.347 (0.048)</b> $\chi^2 = 60.068$ $p < 0.001$	0.268 (0.125)
CHS	0	<b>0.208 (0.080)</b> $\chi^2 = 3.067$ $p = 0.013$	<b>0.711 (0.062)</b> $\chi^2 = 113.574$ $p < 0.001$	CHS	−0.300 (0.191) $\chi^2 = 1.139$ $p = 0.131$	<b>0.192 (0.091)</b> $\chi^2 = 1.876$ $p = 0.049$	<b>1.317 (0.041)</b> $\chi^2 = 9579.851$ $p < 0.001$



**Fig. 3.** Relationship between FCM and cumulative harem size. The relationship between log-transformed faecal cortisol metabolite (FCM) concentrations and log-transformed cumulative harem size ( $n = 135$  observations of 50 stags). Figure shows the raw data, with a fitted line from the regression of log-transformed FCM against log-transformed cumulative harem size.

In concurrence with previous rat (see Sapolsky, 1991 for review) and human (van Cauter et al., 1996) studies, cortisol concentrations increased linearly with age in this population. Laboratory experiments in rats have shown that older individuals take longer to return to baseline levels after a stressor, leading to prolonged periods of cortisol hyper-secretion (Sapolsky et al., 1984, 1986). The age-related increase in FCM levels observed in our study appears to be a consequence of within-individual change (rather than change at the population level), as age at final sampling had no effect on FCM levels. This suggests that the observed age-related variation does not reflect the selective disappearance of particular hormone phenotypes with age (van de Pol and Verhulst, 2006). After accounting for age and sample month, FCM levels were also found to be repeatable, with among-individual differences accounting for around 20% of the total phenotypic variance of this trait after correcting for the fixed effects. Thus those with relatively high FCM concentrations at one sampling point also had relatively high FCM concentrations at other sampling points (and vice versa for low FCM males). This concurs with previous findings of among-individual variance in glucocorticoid metabolite concentrations across a period of several months in wild greylag geese (*Anser anser*) (Kralj-Fisher et al., 2007), although we show this variance to remain across several years.

In this study, males investing the greatest effort in reproduction (in terms of greatest cumulative harem size) were also likely to be

those with the highest baseline FCM concentrations at both the among- and within-individual levels. Stags with relatively high average FCM, therefore, also had relatively larger cumulative harem sizes, and, within individuals' life times, years with relatively high FCM were associated with relatively high cumulative harem size. In red deer, resources such as reproduction (Clutton-Brock et al., 1982) and high quality food (Appleby, 1980; Lincoln et al., 1972) are monopolised by socially dominant stags. Stags compete throughout the year for access to these resources, with high ranking individuals involved in more agonistic and aggressive interactions as a result (Clutton-Brock et al., 1982; Lincoln et al., 1972). Indeed, experimental studies show that reducing aggression through castration causes males to drop in social rank (Lincoln et al., 1972). Research also suggests that high dominance is conserved across the year, with males who dominate in bachelor herds (i.e. male groups outside of the rut), maintaining their high rank in the subsequent rutting season (Clutton-Brock et al., 1982). Given the positive relationship between aggression and glucocorticoid levels observed in other systems (e.g. Muller and Wrangham, 2004), our results support the hypothesis that whilst social dominance enables a high investment in reproduction, it also has associated behaviours (such as agonistic interactions) which lead to corresponding high levels of FCM. This relationship can also be seen within individuals (Table 2(b)): stags had higher FCM levels in years when they invested more reproductive effort (i.e. had larger cumulative harem sizes) than in years when they invested less. Whilst we are unable to comment on the longer-term associations between cortisol and fitness beyond that of a single year, these results do not support the hypothesis that cortisol will negatively influence a stag's reproductive effort within the year of sampling.

## 6. Conclusion

In summary, both faecal androgen and cortisol metabolite (FAM and FCM) concentrations varied with age, and showed pronounced seasonal cycles, with both hormones peaking during the rutting season. Only FCM concentrations were repeatable among individuals; after correcting for age- and season-related variation, FAM concentrations showed no among-individual variance. Males investing more effort during the rut (i.e. greater cumulative harem size) had higher cortisol concentrations than those investing less effort. Given that stags with large cumulative harem sizes tend to be more dominant, this relationship with FCM may be the consequence of more aggressive encounters and effort invested in maintaining their dominance status. Importantly, these results also show that high baseline cortisol levels do not negatively affect a

stag's reproductive effort, and thus opportunity, within the year of sample.

## Acknowledgments

We thank Tim Clutton-Brock for his long-term contributions to the Rum red deer study and for useful comments and discussion. We also thank Scottish Natural Heritage (SNH) for permission to work on the Isle of Rum NNR, as well as the SNH staff on Rum and the Rum community for their support and assistance. We are indebted to the field assistants, volunteers and colleagues who have helped with data collection, particularly Kathi Foerster, Alison Morris, Sean Morris, Martyn Baker, Bruce Boatman and Fiona Guinness. We are also grateful to the Rum red deer group for useful discussion. This research was supported by a Natural Environmental Research Council (NERC) research grant to LK, JP and T. Clutton-Brock, a NERC PhD studentship to AP, a NERC post-doctoral research fellowship to CAW, and an Australian Research Council Future Fellowship to LK.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ygcen.2015.07.009>.

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